



TITLE:

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## Full paper

# GPR40/FFAR1 deficient mice increase noradrenaline levels in the brain and exhibit abnormal behavior



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## ABSTRACT

The free fatty acid receptor 1 (GPR40/FFAR1) is a G protein-coupled receptor, which is activated by long chain fatty acids. We have previously demonstrated that activation of brain GPR40/FFAR1 exerts an antinociceptive effect that is mediated by the modulation of the descending pain control system. However, it is unclear whether brain GPR40/FFAR1 contributes to emotional function. In this study, we investigated the involvement of GPR40/FFAR1 in emotional behavior using GPR40/FFAR1 deficient (knockout, KO) mice. The emotional behavior in wild and KO male mice was evaluated at 9–10 weeks of age by the elevated plus-maze test, open field test, social interaction test, and sucrose preference test. Brain monoamines levels were measured using LC–MS/MS. The elevated plus-maze test and open field tests revealed that the KO mice reduced anxiety-like behavior. There were no differences in locomotor activity or social behavior between the wild and KO mice. In the sucrose preference test, the KO mice showed reduction in sucrose preference and intake. The level of noradrenaline was higher in the hippocampus, medulla oblongata, hypothalamus and midbrain of KO mice. Therefore, these results suggest that brain GPR40/FFAR1 is associated with anxiety- and depression-related behavior regulated by the increment of noradrenaline in the brain.

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## 1. Introduction

The brain contains abundant fatty acids, which serve as constituents of membranes and as an energy source. In addition, fatty acids and their metabolites contribute to signal transduction between neurons or neurons and glial cells, suggesting that fatty acids play a crucial role in development and functional maintenance in the central nervous system (1). It is well known that

polyunsaturated fatty acids (PUFA), including arachidonic acid (ARA; 20:4  $\omega$ -6) and docosahexaenoic acid (DHA; 22:6  $\omega$ -3), are associated with cognitive and emotional function in healthy or pathological conditions (2,3). For instance, the nutritional deficiency of these fatty acids of rodents causes decrease in learning performance and vulnerability to stress which is susceptible to the development of emotional dysfunction (4). Similarly, alterations in PUFA levels in the plasma and brain are observed in subjects with neuropsychiatric disorders (5). However, although fatty acids have become increasingly important for the modulation of neuronal function in the brain, the molecular mechanisms remain unknown.

The free fatty acid receptor 1 (GPR40/FFAR1) is a G protein-coupled receptor, which is activated by long chain fatty acids, such as ARA and DHA (6,7). Accumulating evidence has demonstrated that GPR40/FFAR1 plays a crucial role in the regulation of glucose homeostasis mediated by the free fatty acid-induced

**Abbreviations:** ARA, arachidonic acid; DHA, docosahexaenoic acid; GPR40/FFAR1, free fatty acid receptor 1; EPM, elevated plus-maze test; PUFA, polyunsaturated fatty acid; EPM, elevated plus-maze test; OF, open field; SI, social isolation.

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potentiation of insulin secretion (8). In addition to the regulation of endocrine function, GPR40/FFAR1 is involved in bone remodeling, taste preference for fatty acids, and inflammation (9–11). These findings suggest that free fatty acids exert their physiological functions by the activation of GPR40/FFAR1.

Recent studies have indicated that GPR40/FFAR1 contributes to physiological function in the central nervous system. We have previously demonstrated that activation of brain GPR40/FFAR1 exerts an antinociceptive effect mediated by the modulation of the endogenous opioid system or monoamine system, which is known as the descending pain control system (12,13). We found that activation of GPR40/FFAR1 facilitates the release of endogenous opioid peptides and activates noradrenergic and serotonergic neurons in the brain. In addition to pain control, it is well known that brain opioids and the monoamine system are associated with the regulation of emotional behavior (14,15). Moreover, it has been reported that brain GPR40/FFAR1 signaling is associated with post-ischemic hippocampal neurogenesis in primates (16). Similarly, Zamarbide et al. reported that GPR40/FFAR1 is expressed in the hippocampus and cortex of mice (17). Therefore, it is possible that brain GPR40/FFAR1 signaling regulates emotional and cognitive function. Previously, we found that repeated activation of brain GPR40/FFAR1 decreases immobility behavior in the forced swim test (18), although the involvement of brain GPR40/FFAR1 in emotional behavior remains unclear.

In this study, we analyzed the emotional behavior of GPR40/FFAR1 deficient male mice (GPR40/FFAR1 KO mice) using the elevated plus-maze test (EPM), open field test, social interaction test, and sucrose preference test. Moreover, we evaluated the brain monoamine levels to clarify the relationship between abnormal behavior and molecular change in GPR40/FFAR1 KO mice.

## 2. Materials and methods

### 2.1. Animals

Adult C57BL/6J male mice (7–8 weeks old) were obtained from SLC (Hamamatsu, Japan). GPR40/FFAR1 KO mice were generated by homologous recombination. Exon 1 of the *Ffar1* gene was replaced with a PGK-neo cassette. Frozen *Ffar1*<sup>−/−</sup> fertilized oocytes were inoculated into pseudopregnant foster mothers (ICR). GPR40/FFAR1 KO mice were maintained by crossbreeding homozygous mice. All mice were housed 2–5 per cage with the same sex under standard conditions [23–24 °C, 12 h light/dark cycle (lights on from 8 a.m. to 8 p.m.)]. Food and water were available *ad libitum*. This study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals adopted by the Japanese Pharmacological Society. The Ethical Committee for Animal Experimentation of Kobe Gakuin University approved all experiments (approval number: A16-23; Kobe, Japan).

### 2.2. Experimental design

Fig. 1 shows the experimental design for the behavioral test. The emotional behavior in wild and GPR40/FFAR1 KO mice was

evaluated using EPM, open field, social interaction, and sucrose preference tests at 9–10 weeks of age. All experimental mice were subjected to all the behavioral tests according to the schedule shown in Fig. 1. Each behavioral test was performed as one test per day. After the social interaction test, mice were habituated in two bottle conditions. The behavioral tests were performed during the light phase. Data collection was blinded to the genotype condition.

### 2.3. EPM test

The EPM test was performed as previously described (19). The plus-maze consisted of two open arms and two enclosed arms (both 25 cm in length and 8 cm in width). The arms extended from a central platform (8 cm in length and 8 cm in width). The maze was elevated 50 cm above the floor. The illumination levels on the open and enclosed arms were similar (approximately 60 lux). Mice were placed on the elevated plus-maze for 5 min and their behavior was observed using a web camera. The total number of entries was calculated as the number of entries into the four arms. The entries into open arms were quantified as a percentage of the number of entries into open arms to the total number of entries into four arms. The time spent in open arms was quantified as a percentage of the time spent in all four arms.

### 2.4. Open field test

Mice were placed in a gray open field (45 × 45 cm) surrounded by a 35 cm high wall for 5 min. The camera was positioned 100 cm above the center of the open field. The illumination level on the open field was approximately 6 lux. The total travel distance and time spent in the total area and a delineated center zone (27 × 27 cm) were assessed using a video tracking system (ANY-maze, Neuroscience Inc., Tokyo, Japan). The time spent in the central zone was quantified as a percentage of the time spent in the total area.

### 2.5. Social interaction test

The social interaction test was performed as previously described (19). Experimental mice were placed in the open field (45 × 45 cm) for 2.5 min with an empty perforated plastic box (8 × 8 cm) located at one end in the absence of unfamiliar wild male mice (no target session). The illumination level on the open field was approximately 60 lux. After the no target session, experimental mice were returned to their home cage, and unfamiliar mice were placed in the perforated plastic box. Experimental mice were placed in the open field for 2.5 min in the presence of unfamiliar mice (target session). After the target session, experimental and unfamiliar mice were returned to their home cages. The time spent in the interaction zone surrounding the plastic box was measured during both the no target and target sessions. Social interaction behavior was assessed using a video tracking system (ANY-maze, Neuroscience Inc., Tokyo, Japan), and was quantified as the time spent in the interaction zone in both the no target and target phases and as a ratio of the time spent in the interaction zone in the target

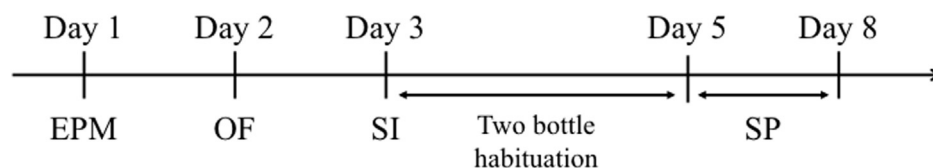


Fig. 1. Experimental schedule. EPM, elevated plus-maze test; OF, open field test; SI, social interaction test; SP, sucrose preference test.

phase divided by the time spent in the interaction zone in the no target phase.

## 2.6. Sucrose preference

The sucrose preference test was performed as previously described (19). Bottles (230 mL) with stoppers fitted with ball-point sipper tubes were filled with a 1% sucrose solution or tap water. First, mice were habituated to drinking from two bottles of tap water for 2 days. Next, they were given a free choice of 1% sucrose solution or tap water. The positions of the two bottles were switched every 24 h, and the sucrose and tap water intakes were assessed once per day and estimated by weighing the bottles. The sucrose preference was calculated as a percentage of total liquid intake and was averaged over 3 days of testing.

## 2.7. LC–MS/MS analysis of monoamines in the each brain area of the mouse

The LC–MS/MS analysis of monoamines in the mouse hippocampus, medulla oblongata, midbrain and hypothalamus was performed as previously described (13). The hippocampal monoamines were extracted using a Monospin PBA column (GL Sciences Inc., Tokyo, Japan). Each level of hippocampal monoamine was corrected for the tissue weight and for isoproterenol level (Tokyo Chemical Industry, Tokyo, Japan), which was used as the internal standard. The results were expressed as the percentage of the content of wild mice.

## 2.8. Statistical analysis

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Significant differences were determined by a two-tailed Student's *t*-test (for two-group comparisons). The time spent in the interaction zone in the social interaction test was assessed using two-way repeated-measures ANOVA followed by Bonferroni's post hoc test. Values of  $P < 0.05$  were regarded as significant.

## 3. Results

### 3.1. GPR40/FFAR1 KO mice showed anti-anxiety-like behavior in the EPM test

The total number of entries into four arms was comparable between wild (15.0  $\pm$  1.2 counts) and GPR40/FFAR1 KO (13.2  $\pm$  1.6 counts) mice (Fig. 2A). The number of entries into open arms (%) was significantly increased in GPR40/FFAR1 KO (40.2  $\pm$  3.9%) mice compared with wild (14.8  $\pm$  3.1%) mice (Fig. 2B,  $P < 0.01$ ). Moreover,

GPR40/FFAR1 KO mice spent a longer time in open arms than wild mice (wild; 6.4  $\pm$  1.7%, KO; 17.0  $\pm$  3.4%, Fig. 2C,  $P < 0.05$ ).

### 3.2. GPR40/FFAR1 KO mice showed normal locomotor activity and anti-anxiety-like behavior in the open field test

For total distance, there was no significant difference between wild (24.7  $\pm$  2.7 m) and GPR40/FFAR1 KO (21.4  $\pm$  1.0 m) mice (Fig. 3A). GPR40/FFAR1 KO (15.8  $\pm$  1.2%) mice spent more time in the central zone compared with wild (10.0  $\pm$  1.9%) mice (Fig. 3B).

### 3.3. GPR40/FFAR1 KO mice showed normal social behavior in the social interaction test

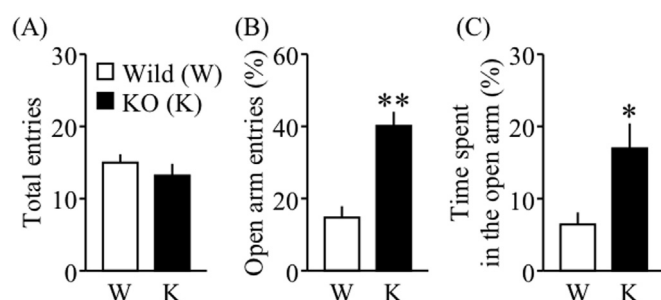
GPR40/FFAR1 KO mice did not show abnormal locomotor activity during the no target and target sessions (wild: no target 11.0  $\pm$  1.1 m, target 7.4  $\pm$  0.7 m; KO: no target 11.8  $\pm$  1.2 m, target 7.2  $\pm$  0.8 m; Fig. 4A). In the social interaction test, the time spent in the interaction zone significantly increased in the target session in both wild and GPR40/FFAR1 KO mice [Fig. 2D; time effect:  $F(1,16) = 34.23$ ;  $P < 0.01$ ], whereas there was no significant effect of genotype [genotype effect:  $F(1,16) = 0.1382$ ,  $P > 0.05$ ]. In the social interaction ratio, there was no significant difference between wild (251.0  $\pm$  37.0%) and GPR40/FFAR1 KO (319.2  $\pm$  96.0%) mice (Fig. 4C).

### 3.4. GPR40/FFAR1 KO mice showed reduction in sucrose preference and intake in the sucrose preference test

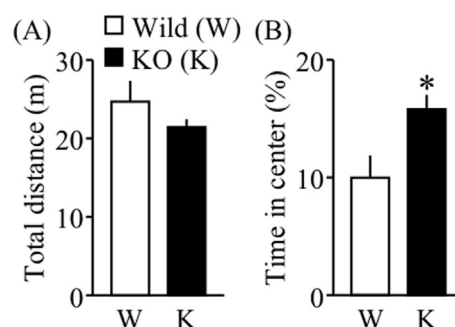
The preference for sucrose was significantly decreased in GPR40/FFAR1 KO (85.2  $\pm$  1.2%) mice compared with wild (88.9  $\pm$  1.1%) mice (Fig. 5A,  $P < 0.05$ ). The total intake of water and sucrose was significantly decreased in GPR40/FFAR1 KO (3.7  $\pm$  0.1 mL) mice compared with wild (4.4  $\pm$  0.1 mL) mice (Fig. 5B,  $P < 0.01$ ). Although the intake of water was comparable between wild (0.48  $\pm$  0.04 mL) and GPR40/FFAR1 KO (0.55  $\pm$  0.04 mL) mice, GPR40/FFAR1 KO mice showed a decreased intake of sucrose (wild; 3.9  $\pm$  0.2 mL, KO; 3.2  $\pm$  0.1 mL, Fig. 5C and D,  $P < 0.01$ ). Moreover, the ratio of sucrose intake divided by weight was significantly decreased in GPR40/FFAR1 KO (0.135  $\pm$  0.004 mL/g body weight) mice compared with wild (0.169  $\pm$  0.006 mL/g body weight) mice (Fig. 5E).

### 3.5. GPR40/FFAR1 KO mice had higher noradrenaline levels in the each brain area

The level of noradrenaline, but not serotonin and dopamine, was significantly increased in the hippocampus, medulla oblongata, midbrain and hypothalamus of GPR40/FFAR1 KO mice compared

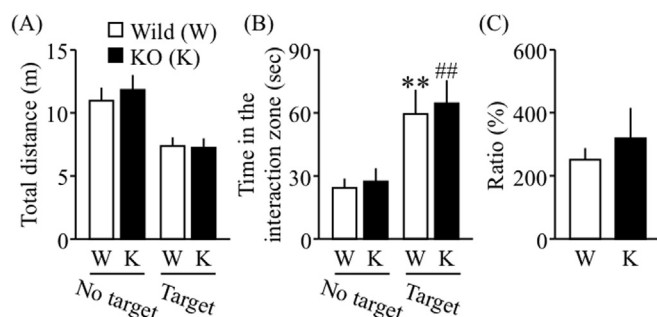


**Fig. 2.** Effect of GPR40/FFAR1 deficiency on anxiety-like behavior in the elevated plus-maze test. (A) Total entries (B) Number of entries into open arms. (C) The time spent in open arms. Data are expressed as mean  $\pm$  SEM. Wild mice:  $n = 10$ ; KO mice:  $n = 9$ . \* $P < 0.05$ , \*\* $P < 0.01$  vs. wild. Student's *t*-test.

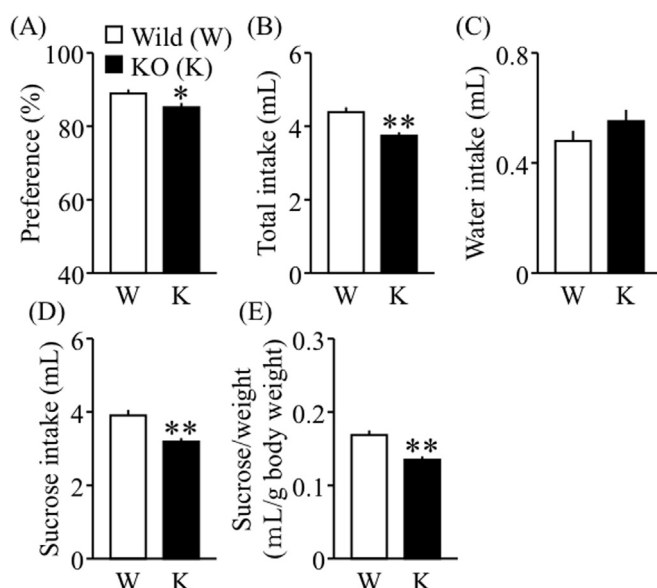


**Fig. 3.** Effect of GPR40/FFAR1 deficiency on locomotor activity and anxiety-like behavior in the open field test. (A) Total distance (m) for 5 min. (B) The time spent in the central zone (%). Data are expressed as mean  $\pm$  SEM. Wild mice:  $n = 10$ ; KO mice:  $n = 9$ .





**Fig. 4.** Effect of GPR40/FFAR1 deficiency on social behavior in the social interaction test. (A) The total distance (m) and (B) time spent in the interaction zone (sec) during no target and target sessions. (C) The ratio of the interaction zone. Data are expressed as mean  $\pm$  SEM. Two-way analysis of variance followed by Bonferroni's post hoc test. Wild mice:  $n = 9$ ; KO mice:  $n = 9$ . \*\* $P < 0.01$  vs. no target wild. ## $P < 0.01$  vs. no target KO.



**Fig. 5.** Effect of GPR40/FFAR1 deficiency on sucrose preference and intake in the sucrose preference test. (A) Preference for sucrose. (B) Total intake of water and sucrose. (C) Water intake. (D) Sucrose intake. (E) Sucrose intake calculated per body weight. Data are expressed as mean  $\pm$  SEM. Wild mice:  $n = 10$ ; KO mice:  $n = 9$ . \* $P < 0.05$ , \*\* $P < 0.01$  vs. wild. Student's  $t$ -test.

with wild mice (Fig. 6A, noradrenaline: wild  $100.0 \pm 6.4\%$ , KO  $148.6 \pm 11.0\%$ ; Fig. 6B, noradrenaline: wild  $100.0 \pm 8.6\%$ , KO  $157.2 \pm 14.0\%$ ; Fig. 6C, noradrenaline: wild  $100.0 \pm 10.9\%$ , KO  $162.4 \pm 48.0\%$ ; Fig. 6D, noradrenaline: wild  $100.0 \pm 9.9\%$ , KO  $119.1 \pm 14.2\%$ ). Each level of hippocampal serotonin and dopamine was significantly increased in GPR40/FFAR1 KO mice compared with wild mice (Fig. 6A; 5-HT: wild  $100.0 \pm 8.6\%$ , KO  $157.2 \pm 14.0\%$ ; dopamine: wild  $100.0 \pm 4.7\%$ , KO  $223.7 \pm 46.9\%$ ), but not medulla oblongata, midbrain and hypothalamus region.

#### 4. Discussion

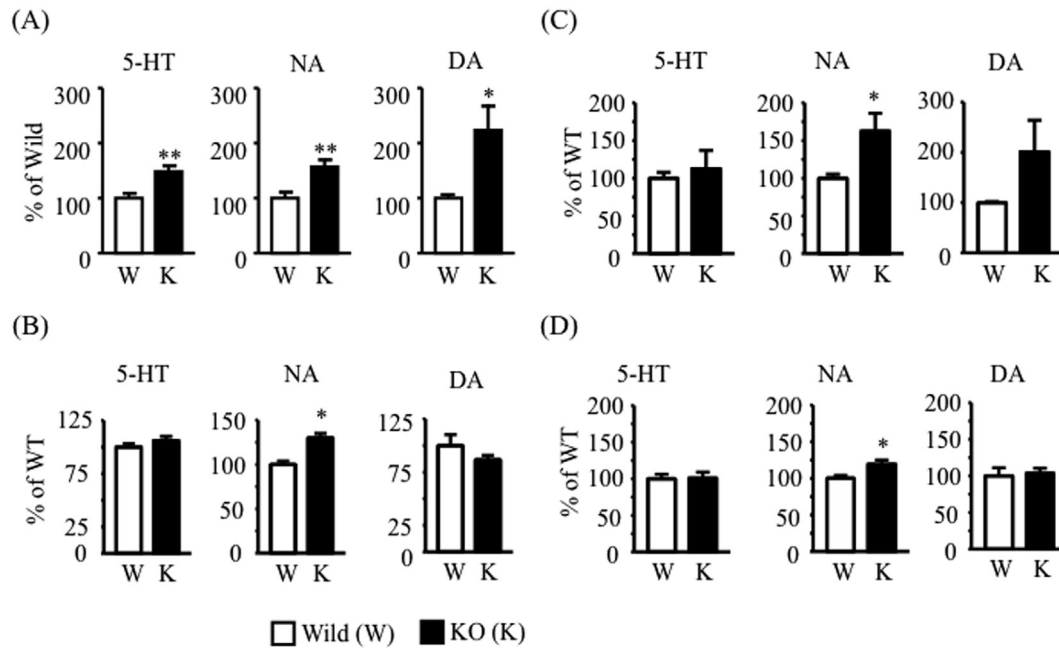
In this study, to investigate the involvement of GPR40/FFAR1 in emotional function, we evaluated the emotional-related behavior of GPR40/FFAR1 KO male mice for the first time. It has been reported that GPR40/FFAR1 KO mice show loss of increased glucose-stimulated insulin secretion induced by free fatty acids and reduced bone density (8,20), indicating the possibility that non-emotional

components induced by the deficiency of GPR40/FFAR1 affect emotional behavior. However, GPR40/FFAR1 KO mice showed normal locomotor activity, which provided important information about interpretation of the results of the behavioral testing. Moreover, it has been reported that GPR40/FFAR1 KO mice show normal regulation of energy balance and food intake (21,22). Therefore, we speculated that the emotional behavior of GPR40/FFAR1 KO mice was appropriately evaluated using behavioral test without the influence of body activity and other non-emotional factors. Moreover, these findings suggest that GPR40/FFAR1 is not involved in the regulation of locomotor activity. However, we cannot exclude the possibility that the sensory impairments (visual, olfactory) could affect behavior of knockout animals in this study.

Both anxiety-like behavior and sucrose preference/intake were reduced in GPR40/FFAR1 KO male mice, while social behavior was normal in KO mice. These findings indicate that brain GPR40/FFAR1 is involved in the modulation of anxiety- and depression-like behavior in rodents. Previous studies have demonstrated that emotional behavior is regulated by fatty acids, which are the endogenous ligands that bind to GPR40/FFAR1 (1,3). For instance, short or long term exposure to a high-fat diet containing higher levels of saturated fatty acids leads to alteration of anxiety-like behavior, locomotor activity, and social interaction (23,24). Similarly, dietary intake or deprivation of  $\omega$ -3 PUFA affects several emotional behaviors, suggesting that fatty acids play a crucial role in the regulation of emotional function (25,26). Accumulating evidences have demonstrated that GPR40/FFAR1 has a role as a sensor in mediating the action evoked by fatty acids. Therefore, these findings suggest that dietary fatty acid-induced alteration of some emotional behaviors is mediated by the modulation of GPR40/FFAR1 signaling in the brain. Furthermore, endogenous ligands for GPR40/FFAR1, such as ARA and DHA, are essential components that are associated with the development of the central nervous system (27,28). Therefore, it is possible that GPR40/FFAR1 deficiency affects the development of the central nervous system and instinctive behavior.

In EPM and open field tests, GPR40/FFAR1 KO mice showed reduced anxiety-like behavior. It has been reported that fatty acids can regulate anxiety-like behavior. For example, systemic administration of palmitic acid, which is a long chain saturated fatty acid, has been shown to increase anxiety-like behavior (29), indicating that increased levels of free fatty acid in the plasma cause an anxiogenic effect by activation of GPR40/FFAR1 signaling in the brain. Consistent with these findings, this study revealed that deficiency of GPR40/FFAR1 signaling produces an anxiolytic effect. It has been reported that mice deficient in fatty acid binding protein 7 (FABP7), which belongs to a family of intracellular lipid binding proteins, showed abnormal composition of brain fatty acid and increased anxiety-like behavior (30), indicating that the development of an anxiety disorder is associated with the abnormal state of brain fatty acids. On the other hand, EPM was utilized to measure impulsive-like behaviors based upon the percentage of time spent in the open arms of the maze (31). GPR40/FFAR1 KO mice showed the number of entries into open arms and spent a longer time in open arms, indicating that GPR40/FFAR1 KO mice may promote impulsivity-like behavior. Therefore, these findings, including our results, suggest that the abnormal function of GPR40/FFAR1 signaling contributes to the pathological mechanism of anxiety or impulsivity-like behavior.

Furthermore, we found that GPR40/FFAR1 KO mice show reduced sucrose preference and intake. Generally, the sucrose preference test is used to evaluate depression-like behavior, which is characterized as anhedonia (32). We have previously demonstrated that GPR40/FFAR1 is localized in the hypothalamic proopiomelanocortin-expressing neurons and is involved in the secretion of  $\beta$ -endorphin, which is one of the endogenous opioid



**Fig. 6.** GPR40/FFAR1 KO mice showed increased monoamine levels in the hippocampus. (A) hippocampus, (B) midbrain, (C) hypothalamus and (D) medulla oblongata. Each level of brain monoamine was expressed as the percentage of content of wild mice. Data are expressed as mean  $\pm$  SEM. Wild: n = 12; KO: n = 12. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. wild. Student's  $t$ -test. 5-HT; serotonin, NA; noradrenaline, DA; dopamine.

peptides (33). It is well known that sucrose intake can release opioids, including  $\beta$ -endorphin, and is suppressed by treatment with opioid receptor antagonists (34,35). Therefore, these findings suggest that the decreased sucrose intake in GPR40/FFAR1 KO mice is associated with  $\beta$ -endorphin release. Because there is no study suggesting that sucrose can activate GPR40/FFAR1 signaling, sucrose intake may indirectly release  $\beta$ -endorphin, which is mediated by brain GPR40/FFAR1 signaling.

Next, GPR40/FFAR1 KO mice had increased monoamine levels in the hippocampus. Hippocampus is one of the most highly connected regions of the brain and function as a brain integrator of emotion and cognition (36). Femenía et al. showed that serotonergic, noradrenergic and dopaminergic neuron project to hippocampus area (37). It has been reported that GPR40/FFAR1 is widely expressed in the brain of mice and GPR40/FFAR1 agonists activate cultured neurons derived from the hippocampus and cortex (17). Of all, hippocampal GPR40/FFAR1 signaling is associated with neurogenesis mediated by the cAMP response element-binding protein (CREB) pathway, which is also regulated by brain-derived neurotrophic factor (BDNF) (38). Therefore, these findings suggest that the hippocampus is a crucial brain region associated with the physiological function of GPR40/FFAR1. The brain monoamine system plays a central role in the regulation of emotional function. Previous studies have demonstrated that anxiety-like behavior is regulated by the hippocampal monoamine system (39–41). Furthermore, to clarify whether other area of brain may affect by the deletion of GPR40/FFAR1, we tested the changes of monoamine levels in other brain region of GPR40/FFAR1 KO mice. We found that the increment of noradrenaline level in the medulla oblongata, hypothalamus and midbrain was observed in GPR40/FFAR1 KO mice compared to wild mice, but not serotonin and dopamine. We have previously demonstrated that GPR40/FFAR1 is expressed in noradrenergic neurons, and activation of GPR40/FFAR1 increased c-fos, a neuronal activation maker, expression in neurons (13), indicating that GPR40/FFAR1 can regulate the function of noradrenergic and serotonergic neurons. In addition, it has been reported that dietary deprivation of  $\omega$ -3 PUFA impairs brain monoamine systems (42,43). In addition, it

is reported that anxiety disorder including post-traumatic stress disorder (PTSD) (44,45) shows the abnormal noradrenergic system in the brain, suggesting that the increment of noradrenaline in the wide brain area may induce, at least in part, abnormal behavior in GPR40/FFAR1 KO mice. Our findings suggest that GPR40/FFAR1 signaling may contribute to the regulation of monoaminergic system in mice. Furthermore, it is thought that deletion of GPR40/FFAR1 could be induced abnormal development of noradrenergic neuron and might alter emotional behavior mediated by the abnormal state of monoamine systems in the brain.

However, Gingrich and Hen reported that absence of the gene at all stages of ontogenesis of mice may interfere with the normal developmental program and/or the organism may undergo changes in other systems to compensate for gene absence (46). In addition, potentially altered maternal behavior of GPR40/FFAR1 KO dams might have a great impact on the resulting phenotype of adult KO offspring because of significant role of maternal behavior in the expression of a phenotype change in adult mice (47,48). Therefore, we cannot excluded that obtained behavioral and physiological changes can be attributed to above-mentioned factors.

In conclusion, we found that GPR40/FFAR1 contributes to the regulation of anxiety- and depression-related behavior in the central nervous system. Moreover, deficiency of GPR40/FFAR1 induces abnormalities in the monoamine system in the brain. These findings suggest that brain GPR40/FFAR1 signaling is associated with the pathology of neuropsychiatric disorders, including anxiety disorders and depression.

#### Conflict of interest

The authors declare no conflict of interest.

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